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Effects of an environmentally relevant concentration of diuron on oyster genitors during gametogenesis: responses of early molecular and cellular markers and physiological impacts

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ABSTRACT

Genitors of the Pacific oyster *Crassostrea gigas* were submitted during gametogenesis to a short pulse exposure to the herbicide diuron at a realistic environmental concentration. Histological analysis showed no effect of diuron on gametogenesis course, sex ratio and reproductive effort. A non-significant increase in testosterone and progesterone levels was observed in genitors exposed to the herbicide. At cell level, diuron exposure was shown to modulate the phagocytic activity of circulating hemocytes. The results of a transcriptional analysisshowedthat diuronaffectedthe expression of genes belonging to functions known to play a major role during oyster gametogenesis such as gene transcription regulation, DNA replication and repair, DNA methylation and cytokinesis. Taking into account the results we previously obtained on the same genitors, this study showed a negative effect of diuron on oyster reproduction by inducing both structural and functional modifications of the DNA.

KEYWORDS

Oyster ; Pesticide ; Reproduction ; Histology ; Gene expression ; RT-qPCR ; Steroid ; Immunity

INTRODUCTION

Marine organisms living in coastal ecosystems are exposed to a variety of chemical compounds from anthropogenic origins related to agricultural or industrial activities that may impact and weaken the populations. Since the last decades, mass mortalities are regularly observed worldwide among marine invertebrates (Harvell et al. 1999), including the Pacific oyster *Crassostrea gigas*, a mollusk species living in intertidal zones and subjected to aquaculture production. Oyster farming industries are suffering from recurrent oyster mortality outbreaks with heavy socio-economical consequences on their activity. Oyster mortality is thought to be explained by complex interactions between oysters (its genetics and physiological status), microorganisms including potential pathogens, and the environmental parameters such as temperature (Samain et al. 2007; Schmitt et al. 2012; Petton et al. 2013). In Europe, pluri-disciplinary research projects were conducted on this issue on both Marennes Oleron Basin in France (MOREST project) and Erbro River Basin in Spain (SCARCE and CEMAGUA projects) (Samain et al. 2007). Among findings, oysters were shown to be particularly vulnerable to physiological and environmental stressors during gametogenesis, when energy is particularly allocated to reproduction. Besides, water quality appears to be a critical point. The presence of chemical pollutants such as pesticides is particularly investigated for the risk it may represent for oyster health. Among pesticides, herbicides are frequently detected in oyster production basin, reaching coastal waters from exoreic catchment area with agricultural activities (Munaron 2004; Soletchnik et al. 2005; Buisson et al. 2008; Burgeot et al. 2008; Caquet et al. 2013). There is therefore a need for a more efficient assessment of the environmental risks associated with the presence of pesticides, which represent 20 out of the 45 dangerous substances identified by the European Water Framework Directive (2013/39/UE). Among substances, diuron (3-[3,4-dichlorophenyl]-1,1-dimethylurea) is a substituted urea compound registered as a pre- and post-emergent herbicide selective for broadleaf weeds and some annual grasses. Despite restriction of its use in France since 2008 (JO no. 204 September 4, 2007), diuron is still detected at high concentrations in coastal waters (Munaron et al. 2012; Buisson et al. 2008; Caquet et al. 2013) due to its fairly persistence in aquatic systems (Thomas et al. 2002). Moreover, diuron has already been reported as having diverse toxic effects on oysters, including genotoxicity in somatic and gamete cells (Bouilly et al. 2007; Akcha et al. 2012; Mai et al. 2013), embryotoxicity (Akcha et al. 2012; Mai et al. 2013) and immunotoxicity (Gagnaire et al. 2006, 2007; Luna-Acosta et al. 2012). As a consequence, diuron is a valuable substance to consider for analyzing the impact of herbicides on marine organisms. Despite an increasing interest on the subject over the last years, there is still a need to determine the toxicity of pesticides under environmentally realistic scenarios of exposure, considering potential trans-generational effects.

In this attempt, a recent study has been undertaken by exposing *C. gigas* oyster genitors to diuron during gametogenesis and investigating the toxic effects in both adults and their offspring. Thus, we have already demonstrated a genotoxic trans-generational effect of diuron by detecting primary DNA damage in both somatic, germinal, and reproductive cells of oyster genitors as well as chromosomal abnormalities in the offspring (DNA aneuploidy) (Barranger et al. 2014, 2015). Moreover, DNA damages were concomitantly observed with a reduced growth and also with developmental abnormalities in larvae from diuron-exposed genitors.

In the present paper, we analyzed the molecular and biochemical effects of diuron on *C. gigas* genitors by measuring the expression of genes relevant to key functions suspected to be affected by pesticide

exposure, the levels of selected enzymatic activities and the activity of immune cell by flow cytometry. Besides, the impact of diuron exposure was also assessed on oyster reproduction process by both qualitative and quantitative histology and steroid quantification in gonads.

MATERIAL AND METHODS

Chemical reagents

Diuron (3-(3,4-dichlorophenyl)-1,1 dimethylurea, CAS number 330-54-1, Pestanal, analytical standard), acetonitrile (anhydrous, 99.8 %), 1-chloro-2,4-dinitrobenzene (CDNB), disodium salt of reduced form of nicotinamide adenine dinucleotide phosphate (NADPH), ultrapure reduced glutathione (GSH), hydrogen peroxide, imidazole, glutamine, and hydroxylamine were purchased from Sigma Aldrich Chemicals (France). Formaldehyde 35 %, glacial acetic acid, and butanol 100 % were purchased from Carlo Erba (France), glycerin 86 % was purchased from Roth (France), ethanol 95 % was purchased from VWR, and paraffin (paraplast®) was purchased from Leica (France). Trizol Reagent and DNAse I were purchased from Invitrogen, M-MuLV Reverse Transcriptase was purchased from BioLabs, and Syber Green Mix was purchased from Roche. Ultrapure deionized water was prepared using a Milli-Q system (Millipore, Molsheim, France). All other reagents were of analytical grade.

Genitor origin and oyster husbandry

As already mentioned in Barranger et al. (2014), the adult oysters were originated from wild genitors collected in the Marennes-Oléron Bay (France). Before the experiment, they were transferred from the field to the hatchery for a 1-month acclimatization period. Seawater was pumped directly from the Seudre river Estuary, filtered through a sand filter (40 µm), and passed through UV rays before draining the tanks in a continuous flow system (output: 55 L h^{-1}). Oysters were fed daily with a mixture of four marine microalgae, and the water temperature was maintained at 8 °C (±1 °C) throughout the acclimatization period. In order to initiate the gonad maturation process, the temperature was then raised by 2° per day for 1 week, to reach 19.8 °C (±0.3 °C) at the start of the experiment.

Diuron exposure during gametogenesis

Once gametogenesis had begun (T0), the oysters were divided into three experimental groups (three 250-L tanks of 240 oysters each per group): a seawater control (SWC), a solvent control (SC, acetonitrile at 0.005 %), and a diuron-exposed (D) group. Two 7-day exposure periods took place at the start and mid-course of gametogenesis. The oysters were maintained in a closed circulation system throughout the exposure period. The seawater was changed every morning, and diuron and acetonitrile were added every day after seawater renewal to reach target concentrations in the tanks. The temperature was kept stable at 20 °C, and oysters were fed the same way as during the acclimatization period. During this experiment, the use of polar organic chemical integrative samplers (POCIS) in control and assay tanks allowed the measurement of both the background level of seawater contamination by pesticides and the reached diuron concentrations. POCIS data demonstrated that oysters were finally exposed to environmentally realistic concentrations of diuron: the concentration reached during the first (Tfp1, 48

h after T0) and second (Tfp2, 5 weeks after T0) herbicide pulse was respectively of 0.2 and 0.3 μg L^{-1} (Barranger et al. 2014). The background contamination of the water used at the hatchery appeared low considering the few data available on the detection of pesticides in coastal water. In the case of diuron, its background concentration was nearly one order of magnitude lower than the one tested in our study.

At Tfp2, oysters from each group were then grown in normal conditions for an additional period of 3 weeks in order to reach maturity (Tp, 9 weeks after T0).

Follow-up of gametogenic cycle and reproductive effort by qualitative and quantitative histology

In order to determine both the sex ratio and gametogenesis stage, 30 oysters were sampled at the beginning and at the end of the experiment (Tp), in each experimental group, just before the onset of spawning. For each sampled individual, transverse sections were realized and fixed for histology in Davidson fluid (glycerin 10 %, formaldehyde 20 %, ethanol 95° 30 %, sterile sea water 30 %, acetic acid 10 %). The tissue samples were dehydrated in serial dilutions of ethanol, transferred in butanol, and embedded in paraffin wax. For each sample, 4-μm slides were prepared and stained according to the Prenant-Gabe trichrome protocol (Gabe 1968). The determination of sex and gametogenesis stage was based on microscopic observation (Nikon Eclipse 80i coupled to a Nikon DXM1200-C camera). Gonad and storage tissue conditions were graded respectively according to Heude Berthelin et al. (2001) and Berthelin et al. (2000). Briefly, gametogenesis stage 0 corresponds to the sexual resting stage. Stage I is defined as the early developmental stage (gonial mitosis) and stage II corresponds to the germinal lineage development. Stage III is characterized as the ripe gonad. Storage tissue development was graded as follows: growing at stage A, dense and structured at stage B, in regression at stage C, and almost completely depleted at stage D.

The reproductive effort was measured at the end of the experiment (Tp) by using 15 slides of the 30 sampled oysters. Slides were scanned with a Nikon Super Coolscan 8000 ED slide scanner provided with a special medical slide holder (FH-8G1, Nikon). The resolution used was 4000 dots per inch (dpi). Total section and gonad surfaces were measured using Vision assistant software. Gonadal index was calculated as the ratio of gonad area over total section area (Royer et al. 2008).

Titration of total steroid levels in oyster gonad

Progesterone (P4), testosterone (T), and estradiol (E2) were measured as described in Dimastrogiovanni et al. (2015). Briefly, gonad samples (0.2–0.5 g wet weight) were homogenized in 2 ml ethanol and frozen overnight at −80 °C. Homogenates were then extracted with 2 mL of ethyl acetate (×3); the organic extract was evaporated under nitrogen, resuspended in 1 mL methanol containing 1 % KOH, and incubated at 45 °C for 3 h. After the saponification step, Milli-Q water (4 ml) was added and the sample extracted with dichloromethane (3×3 mL). The efficiency of the extraction procedure was 65.4±1.3 % for T, 62.4±0.8 % for E2, and 52 ±3.8 % for P4.

Dry extracts were resuspended in 50 mM potassium phosphate buffer pH 7.6 containing 0.1 % gelatin and assayed for P4, E2, and T concentrations using commercial RIA kits. Standard curves with the steroids dissolved in the same phosphate buffer were performed in every run. The limits of detection were 30 pg/g for T, 10 pg/g for E2, and 40 pg/g for P4. Intra-assay coefficients of variations were 14.8

% (T), 12.1 % (E2), and 6.5 % (P4). Inter-assay coefficients of variations were 15 % (T), 11.2 % (E2), and 7.2 % (P4).

Expression profiles of target genes

The expression of 27 genes was analyzed by quantitative RTPCR (qPCR) (Table 1). The basal expression levels of the genes were measured at individual level on whole soft bodies of 15 oysters per group (SWC, SC, D) taken at the end of the second diuron peak. These samples were individually frozen in liquid nitrogen and ground with a Mixer Mill MM 400 (Retsch) under liquid nitrogen conditions. Individual frozen oyster powders were stored at −80 °C until use. Aliquots of 20 mg oyster powders were homogenized in 1 ml of Trizol® reagent (Invitrogen®) for total RNA extraction according to the manufacturer's instruction. RNA samples were treated with DNase I (Invitrogen) to eliminate contaminating genomic DNA and precipitated with 3 M sodium acetate. Total RNA amount and purity were checked by using NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Les Ulis, France) and integrity was analyzed by 1.5 % agarose gel electrophoresis. Fifty nanograms of individual total RNA samples were reversed-transcribed using M-MuLV Reverse Transcriptase according to the manufacturer's protocol (BioLabs®).

qPCR analyses were carried out in duplicate on the LightCycler 480 (Roche) in a final volume of 7.5 µL containing 4.2 µL of reaction Syber Green Mix (Roche), 0.4 µL of each primer (0.6 µM final concentration), and 2.5 µL of individual cDNAs (2 ng of equivalent RNAs). Pipetting steps were performed by the ECHO 525 acoustic liquid handler. Primer sequences used to amplify the 27 selected genes and the three reference genes are shown in Table 1. Cycling qPCR 25 s. At the end of the program, a melting curve of PCR program consisted in a pre-incubation step at 90 °C for product was performed to ensure the production of a single 10 min, followed by 40 cycles of denaturation at 90 °C for specific product. The relative expression was normalized ac10 s, annealing at 57 °C for 20 s, and extension at 72 °C for cording to the ΔΔCt method (Livak and Schmittgen 2001) with the Cq geometric mean value of the eukaryotic translation elongation factor 1-alpha (Cg-ef1α: AB122066), the ribosomal protein L40 (Cg-rpl40: FP004478), and the 40S ribosomal protein S6 (Cg-rps6: CU686508) as endogenous reference genes (Rosa et al. 2012).

Time-course activity of enzymes involved in biotransformation and antioxidant defenses

To assess the time-course activity of glutathion-S-transferase (EC 2.5.1.18), catalase (EC 1.11.1.6), and glutamine synthetase (EC 6.3.1.2), pieces of oyster digestive gland were individually sampled at T0 (24 individuals) and in each experimental group at the beginning and end of the two herbicide pulses (24 per group, 8 in each triplicate tank at Tfp1 and Tfp2). Samples were frozen and stored in liquid nitrogen up to analysis.

Samples were thawed in ice and homogenized in a phosphate buffer (K_2HPO_4/KH_2PO_4 100 mM, pH 7.4, *w/v*=1/3) and a Tris buffer saline (50 mM Tris-HCl pH 7.5, 150 mM NaCl) for CAT and GS, and GST, respectively. Homogenates were centrifuged for 15 min at 9000 g at 4 °C to recover the S9 fractions. The protein concentration of each sample was then measured by a colorimetric Bradford method using Bio-Rad protein assay reagent at 595 nm. The selected activities were then measured in 96-well microplates using a Safire spectrofluorimeter (TECAN).

CAT activity was measured as described by Greenwald (1985), using the decrease in absorbance at 240 nm, due to H_2O_2 consumption. GST activity was measured using 1chloro-2,4-dinitrobenzene (CDNB) as a substrate and the occurrence of GSH-CDNB complex was followed at 340 nm (Habig et al. 1974). GS activity was assayed by the γglutamyl transferase reaction as described by Webb and Brown (1976) and modified by Saha et al. (2002). The formation of γ-glutamyl hydroxamate was followed at 543 nm.

Specific activity was expressed in nanomole per minute per milligram (nmol min^{-1} mg^{-1}) protein for CAT and GS and in micromole per minute per milligram (μmol min^{-1} mg^{-1}) for GST.

Phagocytosis assay

The phagocytic activity of hemolymph cells has been assessed in vitro according to a previously described method (Auffret et al. 2004) applied for the last decade to several bivalve species. Briefly, two 50 μL replicate samples of freshly withdrawn hemolymph (n=12 individuals per group at Tfp1 and Tfp2) were incubated in a 96-well microplate with inert, fluorescent particles (Polysciences, Fluoresbrite™ YG 2.0 μM latex microspheres) at an estimated 1:30 ratio for 4 h at a standard temperature of 15 °C. The incubation medium was removed and the cell suspension fixed with a commercial paraformaldehyde solution (BD, CellFix™) until analysis by flow cytometry (GUAVA EasyCyte™). Parameters used to characterize the phagocytic activity were obtained through data analysis with the GUAVA ExpressPro™ software. For each individual, a phagocytic index (PI, expressed in % positive cells) was calculated as the number of cells having engulfed three beads or more. The normalized phagocytic capacity of positive cells (NPC, expressed in arbitrary units), corresponding to the estimated number of particles internalized per active cell, was calculated from the same histogram of fluorescence intensity distribution (log units) as the geometric meanofthisselectedevent subpopulation. This parameter was normalized with the fluorescence value obtained for single beads as recommended by Lehmann et al. (2000).

Statistics

For gene expression, analyses were done with the R software (v3. 1 64bits) and ddCt package (v1.18.0). For non-normal values, Kruskal-Wallis test was used, followed by a MannWhitney pairwise comparison test. Significant differences were considered at $p<0.05$.

Enzymatic biomarker responses were analyzed using Statistica 8.0 (Statsoft, Inc.). Raw data were first tested for normality (Shapiro test) and homogeneity of variances (Levene test). For normal values, one-way or main effects ANOVAwere used followed by Tukey's pairwise comparison test if required. Results of quantitative histology were compared using a Student's test. Values were significantly different for $p<0.05$.

RESULTS

Diuron exposure at an environmentally realistic concentration did not affect gametogenesis course, sex ratio, reproductive effort, and total steroid levels

Analysis of gonad histology showed that at the beginning of the experiment and so before the first herbicide pulse, most of the oysters already started gametogenesis (stage I or II) (Fig. 1). At the end of the broodstock conditioning, no difference in gametogenesis course was noticed between the control and diuron-exposed oysters. They were all globally mature, reaching stage III of gonad development (Fig. 1). Gametogenesis was synchronous in males and females (Fig. 1). In the control group, a slightly better use of storage tissue for gamete production has been achieved (Fig. 2). From a quantitative point of view, no effect of solvent or diuron was observed on the *sex ratio*, nearly 50/50 whatever the experimental groups. Although a significant decrease in genitor wet weight has been observed during this experiment following diuron exposure (Barranger et al. 2014), no quantitative difference was noticed between the three experimental groups in terms of total slide surface and gonad surface (data not shown). The three experimental groups present the same gonad index at the end of the broodstock conditioning (Fig. 3). A high inter-individual variability in the levels of estradiol, testosterone, and progesterone was observed in gonads, with no clear relationship with sex or gonad development stage (Fig. 4). Concerning the impact of diuron exposure, no effect on steroid levels was observed at Tfp2 in comparison with levels measured in control groups. However, an upward trend was noticed at the time of spawning (Tp) for T and P4 following herbicide exposure, resulting in a 5.5- and 4.5-fold increase in T and P4 levels, respectively. On the other hand, E2 was the only steroid whose level changed over the length of the experiment: a significant decrease in E2 was observed during maturation in both control and assay tanks.

Diuron significantly affected the expression of genes involved in apoptosis, gene transcription and repair, replication and methylation of the DNA

The effect of diuron exposures on the oysters has been analyzed at the transcriptional level. For that, we selected 27 genes according to their broad relevance to function categories that could be affected upon pesticide exposure, namely stress response, xenobiotic biodegradation, antioxidant defenses, and apoptosis (Table 1). Those categories were also DNA methylation, gene transcription regulation, DNA replication, recombination and DNA repair, and cytokinesis that have major functions particularly during gametogenesis (Dheilly et al. 2012).

Among the analyzed genes involved in stress response, xenobiotic degradation or antioxidant activities, no significant differences were seen between the three oyster groups in their expression levels (Fig. 5a–c, respectively). In apoptosis gene category (Fig. 5d), *cytochrome c* expression was increased in oysters exposed to diuron or to the solvent but significantly only in the case of the herbicide exposure ($p = 0.007$). Conversely, oyster exposure to diuron led to a decrease in the expression of caspase 3 that appeared to be significantly downregulated in oysters exposed to the solvent alone ($p=0.005$).

Diuron and solvent exposures affected the expression levels of *DNMT1* and *DNM3A* from DNA methylation function category (Fig. 5e), with significant decrease in *DNMT1* expression in diuron oyster group ($p=0.008$). In gene transcription regulation category (Fig. 5f), gene expression levels were reduced in diuron oyster group, and at a lesser extent in the solvent group, but only *KDM1* was significantly affected by diuron exposure ($p=0.02$). In DNA repair and DNA replication category, diuron was shown to significantly increase the expression level of *XRCC4* ($p=0.03$) whereas those of *CAF1* was significantly decreased ($p=0.04$) (Fig. 5g, h). It is noteworthy that in all three oyster groups, great variability in gene expression was observed in *TOP1* expression from zero to more than 400-fold

changes according to individuals (Fig. 5h). Finally, *PRC1* involved in cytokinesis was observed overexpressed in exposed oysters, but only significantly upon diuron exposure (p=0.02) (Fig. 5i).

No change in the activity of enzymatic biomarkers was observed

No effect of diuron or solvent exposure was shown in the activity of GST, GS, and CAT in oyster genitors (p>0.05). CAT was the only enzyme whose level changed significantly with time but independently from diuron exposure (p<0.001).

Diuron exposure altered the phagocytic activity of oyster hemocytes

The defense status of genitor oysters was assessed by measuring two major parameters describing the phagocytic activity of circulating hemocytes, namely the phagocytosis index, in the all hemolymph cell population and the phagocytic capacity of active hemocytes. At Tfp1, both parameters were found altered by diuron exposure but also by the solvent (Fig. 6). The percentage of active cells (phagocytosis index) dropped from 71.9 to 55.8 and 60.0 %, respectively (p< 0.001). Exposure to diuron had a stronger effect on the phagocytic capacity of hemocytes compared to both untreated and solvent-exposed (p<0.001). At Tfp2, the mean phagocytic capacity was moderately increased (p=0.11) for diuronexposed oysters (12.1 units) compared to the other treatments (9.7 and 8.4 units for the SWC and SC controls, respectively).

DISCUSSION

Diuron affected the expression of genes belonging to functions known to play a major role during oyster gametogenesis and modulated the phagocytic activity of hemocytes

The effects of pollutant exposure on reproduction are widely studied in ecotoxicology because the effects observed at individual level can bring information on the adverse effects encountered by the whole population at a longer time scale. Since the middle of the 1990s, there is a growing number of papers dedicated to the effect of pollution on animal reproduction. The effects of pollutant exposure on reproduction can be assessed in adults at different levels, from the molecular (modulation of the expression level of genes involved in sex determination, steroid synthesis and regulation,…) to biochemical (titration of sexual steroids, measurement of enzymes involved in steroid synthesis or homeostasis) or tissue levels (histological analysis of the gonad: sex ratio, reproductive effort, gametogenesis kinetics). The impact of pollutant exposure on both gamete viability and functionality, as well as on hatching rate and early developmental stages is also highly relevant to assess adverse effects on reproduction. In addition, the potential ofa substance to be an endocrine disruptor can be easily determined both in vitro and in vivo thanks to the use of specific assays (Clode 2006; Rotroff et al. 2013).

In this context, we have assessed the effect of a pulse exposure to the herbicide diuron on oyster reproduction. At tissue level, no significant effect of diuron on gametogenesis course and reproductive effort has been achieved. In several aquatic species, chemical pollution has already been shown to impair gametogenesis. In the oyster, *Crassostrea angulata*, from different locations in Taiwan, low gonad

maturity index and pathological alterations (e.g., atresia, hemocytic infiltration of gonadal tissue) were associated to chemical pollution, in particular to the presence of elevated heavy metal concentrations (Vaschenko et al. 2013). Recently, in vitro exposure of zebra fish ovary tissue to the herbicide glyphosate (15 days, 65 mg/L) resulted in a significant increase in both oocyte diameter and in the presence of unusual concentric membranes associated with the external membranes of mitochondria and yolk granules (Armiliato et al. 2014). In the present study, the absence of diuron effect on oyster gonad structure could be related to the low concentrations of diuron selected for the experiment as well as to the short duration of the exposure that was limited to only two 7 day-exposure pulses. Moreover, it cannot be excluded that differences may also exist between the different gametogenesis stages in terms of pollutant sensitivity. As a matter of fact, in this study, oyster genitors have been exposed to diuron after the major peak of gonial mitosis when they already started gametogenesis. Because the reproductive effort is highly dependent on the quality of gonial mitosis, it would have been at higher risk to expose the genitors sooner to target this active phase of gametogenesis. Moreover, it would have been interesting to follow up the expression of specific markers of germinal niche to assess the impact of diuron on both the early stages of gametogenesis and germ cell line. These marker genes such as vasa- and piwi-like genes have been previously identified by sequence homology in the oyster but their function still remains to be clarified (Fabioux et al. 2004, 2009; Zhang et al. 2012).

Interestingly, results obtained at molecular level highlighted an impact of diuron exposure on the expression of genes belonging to functions known to play a major role during oyster gametogenesis such as DNA methylation, gene transcription regulation, DNA replication, recombination and DNA repair, and cytokinesis (Dheilly et al. 2012). As a matter of fact, gametogenesis is characterized by a high mitotic activity. As a consequence, cell division and differentiation request mechanisms involved in both gene regulation including epigenetic mechanisms, gene transcription, and DNA replication. In our study, diuron decreased the expression level of DNMT1, KDM1, and CAF1 that are respectively involved in DNA methylation maintenance, gene transcription regulation, and DNA replication. To the opposite, it significantly increased the expression of cytochrome c, XRCC4, and PRC1 genes. Modulation of these latter genes involved in apoptosis, DNA repair, and cytokinesis could indicate a need for maintaining the genome integrity of the cells. Cytochrome c initiates a major caspase activation pathway in response to apoptotic stimuli that can lead to subsequent cell death. Apoptotic cells are known to present a high level of DNA damage that could be repaired in some extent by DNA repair mechanisms. Moreover, the presence of DNA lesions can activate checkpoint pathways that regulate specific DNA repair mechanisms all along the different cell cycle phases, modifying cytokinesis. It is worth mentioning that this assumption is in accordance with the results previously obtained by Barranger et al. (2014) on the genotoxicity of diuron in oyster genitors from the present experiment. They demonstrated the genotoxicity of the herbicide by the detection of structural DNA lesions in both germinal (8-oxodGuo) and gamete (DNA strand breaks) genitor cells, respectively (Barranger et al. submitted; 2014). Moreover, they put forward a decreased in the hatching rate and a higher level of developmental abnormalities when using embryo from diuron-exposed genitors for fecundation (Barranger et al. 2014). Taking into account these latter results, we can so affirm that despite the absence of structural changes in gonad tissue, diuron exposure had a negative effect on gamete quality in terms of DNA integrity and on progeny early performances.

Finally, in our study, an upward trend for testosterone and progesterone levels at time of spawning was observed in diuron-exposed genitors but it was not statistically significant. Because there are

significant evolutionary divergences in endocrine strategies, it remains difficult to transpose knowledge acquired in higher vertebrates to invertebrates. Testosterone, progesterone and estradiol were unequivocally identified in mussels (Reis-Henriques et al. 1990), but it is still difficult to conclude that these steroids function as in invertebrates (Scott 2013). In fish, estrogens play a role in the regulation of gonadal sex differentiation and sex change in most teleost fishes (Nakamura et al. 2003; Guiguen et al. 2010). In the present study, a significant decrease of E2 content in gonads was observedovermaturation, which agreeswith the steroidogenic shift from E2 to progestagens, occurring in fish ovarian follicles immediately prior to oocyte maturation, which is needed for the growing oocytes to enter the maturation stage. Such as in fish, progesterone could be involved in oyster oocyte maturation and germinal vesicle breakdown (Dimastrogiovanni et al. 2015).

In addition to the molecular effect of diuron on gametogenesis, herbicide exposure was shown to reduce the phagocytic capacity of hemolymph cells, a parameter linked to hemocyte competence, after the first exposure pulse when the number of active cells remained slightly altered. Herbicides appear as substances able to modulate the immune status of aquatic invertebrate species, at environmental levels within few days in adult oysters (Gagnaire et al. 2007) or within hours in young individuals (Luna-Acosta et al. 2012). These responses observed after the first exposure pulse could be considered as early effects of contamination. The exact mechanisms of action remain unknown and chronic exposure of Pacific oysters to diuron (Bouilly et al. 2007) did not show any dose-effect relationship, indicating possible indirect consequence of other physiopathological, systemic alterations. Stress-induced immunomodulation has been reported in several groups of mollusk species as abalones (Hooper et al. 2007) and oysters (Lacoste et al. 2001). As so-called effect biomarkers, immunotoxic responses are not necessarily induced directly by contaminants but would correspond to some secondary stress response. In the present study, we hypothesize that a « general stress » state was induced upon the first exposure pulse. Observation in these oysters of genotoxic effects (most probably linked to oxidative stress induction) supports this hypothesis (Barranger et al. 2014). Other molecular biomarkers reported after the secondexposurepulse with a higher targeted concentration of pesticides, rather reveal interactions with biological targets in tissues.

Fate of diuron in oyster and detoxification process

The effects of diuron we observe here in oyster genitors at both molecular (gene expression) and cellular (cell immunity) levels complete those results previously obtained by Barranger et al. (2014; submitted) on either genitors (genotoxicity and reduced growth) and their descent (genomic abnormalities, reduced hatching rate and growth, developmental abnormalities). All these effects are thought to be highly dependent from diuron's fate once absorbed in the oyster.

The bioconcentration factor of diuron in oyster was 7 in the study of Buisson et al. (2008) up to 17 in the study of LunaAcosta et al. (2012). These values remained low, indicating a weak ability of diuron to bioaccumulate in oyster tissues. Diuron is hence probably biotransformed into its main metabolites, namely *N*-(3,4-dichlorophenyl)-*N*-methylurea (DCPMU) and 3,4-dichlorophenylurea (DCPU), such as in other organisms like bacteria (Vercraene-Eairmal et al. 2010), rat, dog, and human (Abass et al. 2007). None of these metabolites have been yet detected in the oyster but HPLC MS-MS analysis actually in progress in our laboratory exhibited the ability of oyster to produce both metabolites in vitro (unpublished results). It is worth mentioning that in *C. gigas* exposed to a mixture of pesticides containing glyphosate, its main metabolite named aminomethylphosphonic acid (AMPA) was detected

in oyster tissues, probably resulting from the biotransformation of the parent herbicide in the oyster (Geret et al. 2013). Moreover, the biotransformation products of other pollutants such as the PAHs, benzo(a)pyrene (Anderson 1985) and pyrene (Bustamante et al. 2012) or the pesticides, chlorpyrifos (Woodburn et al. 2003), and pentachlorophenol (Shofer and Tjeerdema 1993) have already been identified in the oyster *Crassostrea virginica* demonstrating the metabolic ability of *Crassostrea* species to biotransform xenobiotics.

Biotransformation relies on different kinds of enzymes involved in oxidation, reduction, and hydrolyse (phase I enzymes) and conjugation with endogenous molecules to increase hydrophilicity (phase II enzymes). Cytochrome P450 and GST are phases I and II enzymes, the more frequently measured to assess pollutant exposure explaining their selection for the transcriptional analysis. In the present paper, the expression levels of these two genes were shown to be nonmodulated by diuron exposure. Because biotransformation is a rapid response to pollutant absorption, sampling may have occurred too late (end of the second herbicide pulse) to detect change in gene expression related to xenobiotic biotransformation. The short exposure pulse at an environmentally concentration can also explain the absence of significant change in gene expression level. Few data are available on the transcriptional analysis of biotransformation genes in the oyster. These last years saw a significant advance in oyster genomic resources (Zhang et al. 2012). A gene belonging to the CYP sub-family, CYP356A1, was identified in oysters exposed to a sanitary sewage-effluent (Rodrigues-Silva et al. 2015). Through the production of a CYP356A1 purified protein and a corresponding mouse polyclonal antibody, dot blot analysis highlighted the inducibility of the CYP proteins in the lining epithelial cells of mantle, stomach, intestine, and gills but also in free cells such as hemocytes. In the species *Crassostrea brasilia*, recent work also indicated the presence of new cytochrome P450 and glutathione-S-transferase genes those expression was upregulated by exposure to phenantrene (Lüchmann et al. 2014). Other genes involved in xenobiotic detoxification process such as those coding the multixenobiotic resistance protein P-170 were also identified in *Crassostrea* species (Zhang et al. 2012; Mottier et al. 2014). These new data will help to better investigate the response of biotransformation genes in the case of pollutant exposure. It is worth mentioning that antioxidant genes such as catalase and superoxide dismutase were also not modulated by diuron exposure. Numerous pollutants including herbicides are known to induce an oxidative stress in the oyster, involving antioxidant defenses. Diuron is thought to induce toxicity in oyster by oxidative stress as suggested by the oxidative DNA damage it induces in the oyster (Akcha et al. 2012; Barranger et al. 2014).

At biochemical level, no effect of diuron exposure was detected on CAT, GST, and GS activities. In the study from Geret et al. (2013), the modulation of these activities was reported in *C. gigas* following exposure to pesticides. However, oysters were subjected to a continuous exposure to a mixture of pesticides (0.8 μgL^{-1} alachlor, 0.6 $\mu g\ L^{-1}$ metolachlor, 0.7 μgL^{-1} atrazine, 0.6 μgL^{-1} terbuthylazine, 0.5 μgL^{-1} diuron, 0.6 μgL^{-1} fosetyl aluminum, 0.05 μgL^{-1} carbaryl, and 0.7 μgL^{-1} glyphosate) and at a concentration higher (4.55 μgL^{-1}) than those tested in the present study (0.2–0.3 μgL^{-1}). For the latter exposure conditions, a significant increase in gill and digestive gland CAT and GS activity was observed. GST was also significantly increased but only in the digestive gland. It is noteworthy that changes in enzymatic activity levels were observed rapidly after the beginning of the experiment. In the case of CAT activity, increase was significant at day 1 and 3 but no longer at day 7. Such as mentioned earlier for the expression of genes involved in biotransformation and antioxidant defenses, the absence of effect could be related to late sampling but also to the short exposure period and weak tested concentration.

CONCLUSION

Short pulse exposure of oyster genitors to diuron during gametogenesis was performed in this study at a concentration realistic in an environmentally point of view. Whereas no significant direct effect of herbicide exposure was observed on oyster steroid levels, gametogenesis course, and reproductive effort, diuron affected the expression of genes belonging to functions known to play a major role during oyster gametogenesis. These functions are DNA methylation, gene transcription regulation, DNA replication, recombination and DNA repair, and cytokinesis. Interestingly, these molecular responses may reflect diuron genotoxicity responsible for the decreased in gamete and progeny quality we reported in diuron-exposed genitors from the same experiment. Short exposure to diuron at an environmental concentration impacted the oyster genitors themselves but also their next generation when exposed at sensitive physiological state such as gametogenesis. Further experiments should explore the mechanisms by which diuron exerts its toxic effect on ongoing gametogenesis. These results address the question of the transgenerational effect of herbicides on germ lineage, fitness, and adaptive capacity of oyster populations in anthropic environment.

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TABLES & FIGURES

Table 1
List of the 27 genes analyzed by quantitative RT-PCR in oyster genitors and their respective primer sequences

Gene name and function	Symbol	GenBank	Forward primer (5′ –	Reverse primer (5′ –
Stress response				
Metallothionein IV	MT	AM265551	CAGCTCACACAGTCCCTTC	CATGTACAGTTACACGATGC
Heat shock protein	HSP74	AAD31042	GCATGTGAGCGAGCAAAACG	TGGCAGCTTGAACAGCAGC
Complement C1q-like protein	C1QL3	EKC35623	GCCAGGAAATCTGAAGAGTG	GTTATCCGACTCAAACAGTG
Universal stress	USP	EKC39971	TTGAGGTTTCCGTGAACGAG	AACAATCACCGGAACTGACG
Xenobiotic biodegradation				
Cytochrome P450-related	CYP	EF451959	AACATCATCGGAATGCT	GATACAGACCGCCGTAA
Glutathione S-transferase	GST	EKC30389	ATGACCTTCCTGAACACTGG	GTTGACCACAAAGGCCACTC
Antioxidant				
Peroxiredoxin	PRDX4	ADC35419	GATCACCATGAATGACCTCC	ACATTGGGACGGATTGTGTC
Probable maleylacetoacetate isomerase	MAAI	ACO15091	GCAGAATGGGCTAAGTTCTG	AGAGTCTAGCTGCTGAACAC
Selenium-dependent glutathione	GPX5	ABS19600	TGCTGTAAACTGACATATGACA	GCCTATCGTTGAGAACCATG
Catalase	CATA	ABS18267	CACCCAGAAGTTGATCCGC	GCTCACATTGCTGACGGAG
Extracellular superoxide dismutase [Cu-	SOD	DQ010420	AGAGGTGAATGCTACCAGG	AGGCCAAGAATTCCGTCTG
Apoptosis				
Mitogen-activated protein kinase kinase kinase	M3K7	EKC20090	CGGAGGAGTTCAAACAACTG	TCGAGTAACGTAGTCGTTGG
Cytochrome c	CYC	EKC38518	GAGGACATCTGATTGCTTGC	TGTTACGGCAAGTCAACTGC
Caspase-3	CASP3	EKC30354	ATCACCAGGAAGGATCATGG	GTTCATCCGAACACGACTCG
DNA methylation				
DNA (cytosine-5)-methyltransferase 1	DNMT1	EKC23761	CCGGAGGATACACACAA	AGTACTCCTCTAGGCTT
DNA methyltransferase 1-associated	DMAP1	EKC31208	GTAGTAGCAAAGTCAGACC	TATACAGGGATGTCTACCG
DNA (cytosine-5)-methyltransferase	DNM3A	EKC24789	TATAGTCAACCCAGCTAGAAG	TGATAGTGTCTCTGATGTCG
Gene transcription regulation				
Lysine-specific histone demethylase	KDM1	EKC24738	CAAAGAGTGTATCAAGGGTA	ATGACCAAACAGGTTAGC
Histone deacetylase	HDAC4	EKC34968	GGTTACAATGTCTCCTCAG	CATCTCCAAGAAGAGCTTTA
Metal-response element-binding transcription	MTF2	EKC42907	CAAGGTCTTGTCAGTTAGG	CAAATGTAATGCAAGAGCAG
DNA recombination and repair				
DNA ligase 4	DNLI4	EKC42907	GAAGCCAACACAAATCAAG	CATCAGTCCTCCCACAT
DNA repair and recombination protein RAD54B	RAD54B	XM_011444080	CAAGGTCTTGTCAGTTAGG	CAAATGTAATGCAAGAGCAG
DNA repair				
DNA repair protein	XRCC4	EKC30529	AGACAGACTATGACAGGAT	TCACCGTTTGCATAGTTAC
DNA replication and repair				
Probable histone-binding protein Caf1 isoform	CAF1	XP_003424838	TGCTTCACATTACGACAGCG	TGTCTTCGTGGCGATGATAC
DNA replication and transcription				
DNA topoisomerase	TOP1	EKC21526	GCAACGAGGAGGTAACAAGG	TATAATCCGCCTTCGCTGTC
DNA replication licensing factor	MCM7	Q6NX31	GCCAGAAATAGCAAGGACACC	TGGGCAGGATTGAGAGAGTC
Cytokinesis				
Protein regulator of cytokinesis	PRC1	O43663	GTGAGAGAAAGTGAAGAGAC	TAGGATGCCACACTGCTGTC
Genes used for normalization				
Elongation factor 1	EF1	EKC33063	GAGCGTGAACGTGGTATCAC	ACAGCACAGTCAGCCTGTGA
Ribosomal protein L40	RPL40	EKC19428.1	AATCTTGCACCGTCATGCAG	AATCAATCTCTGCTGATCTGG
40S ribosomal protein S6	RPS6	AFJ91756	CAGAAGTGCCAGCTGACAGTC	AGAAGCAATCTCACACGGAC

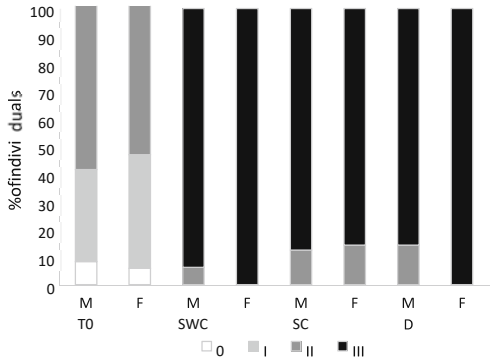

Figure 1

Gametogenesis course in the three experimental groups: sea water control (*SWC*), solvent control (*SC*), and diuron-exposed group (*D*) and for both sexes (M and F). Results were expressed as percentage of oysters at each gametogenesis stage at the beginning of the experiment (T0) and at the end of the breeders conditioning. Stage 0: quiescent phase, stage I: first gonial mitosis, stage II: mitosis and meiosis, stage III: sexual maturity. (n=30)

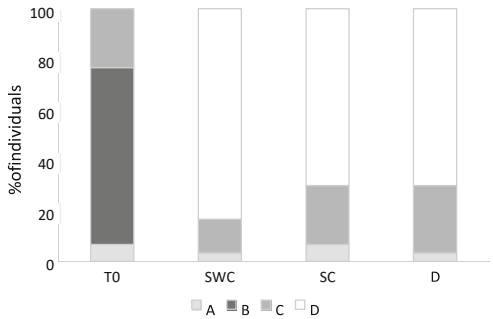

Figure 2

Storage tissue development in the three experimental groups: sea water control (*SWC*), solvent control (*SC*), and diuron-exposed group (*D*). Results were expressed as percentage of oysters at each storage tissue stage at the beginning of the experiment (T0) and at the end of the breeders conditioning. Stage A: phase of restructuring, stage B: maximal storage tissue development, stage C: phase of regression, stage D: declined stage. (*n*=30)

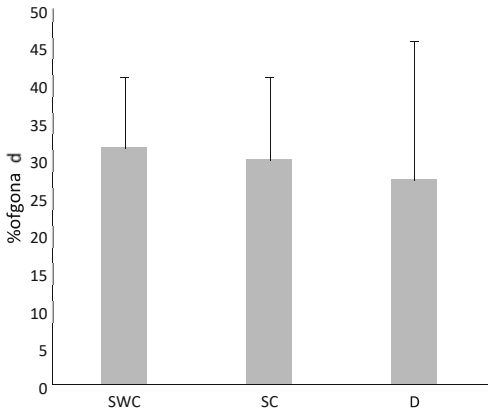

Figure 3
Assessment of oyster reproduction effort using stereological analysis of the relative percentage of gonad in the gonadal tissue area of the three experimental groups: sea water control (*SWC*), solvent control (*SC*), and diuron exposed group (*D*). Results were expressed as percentage of the whole surface animal occupied by gonad. (n=15)

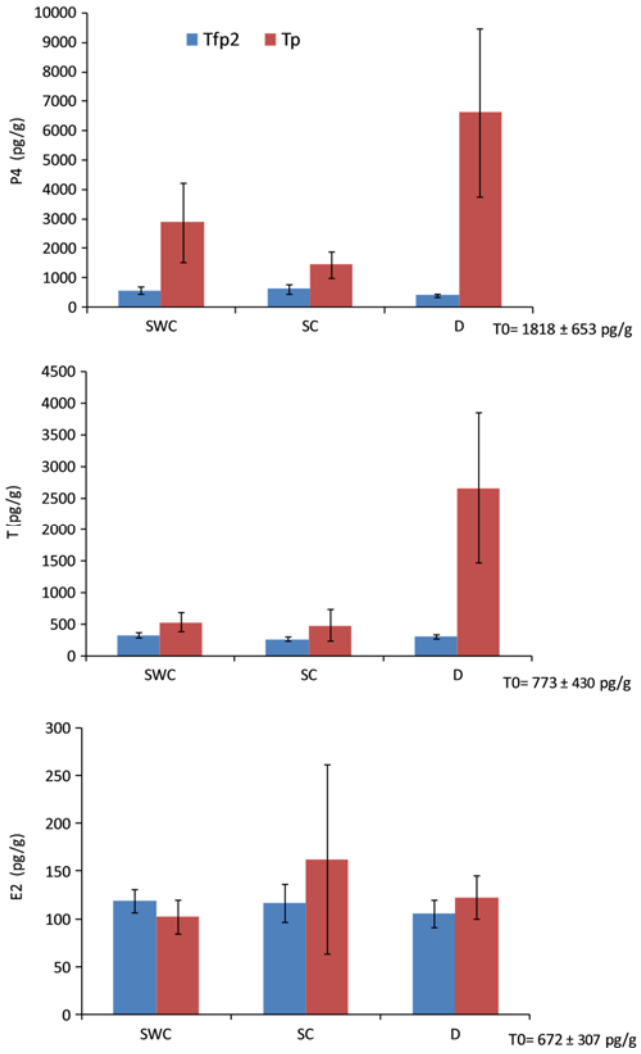

Figure 4
Levels of progesterone, testosterone, and estradiol in gonad tissue of control and diuron-exposed oysters at Tfp2 and Tp. Concentrations determined in control oysters at time 0 are indicated in the bottom of the figures. SWC: control; SC: solvent control; D: diuron. Data expressed as mean±SEM (*n*=6–9)

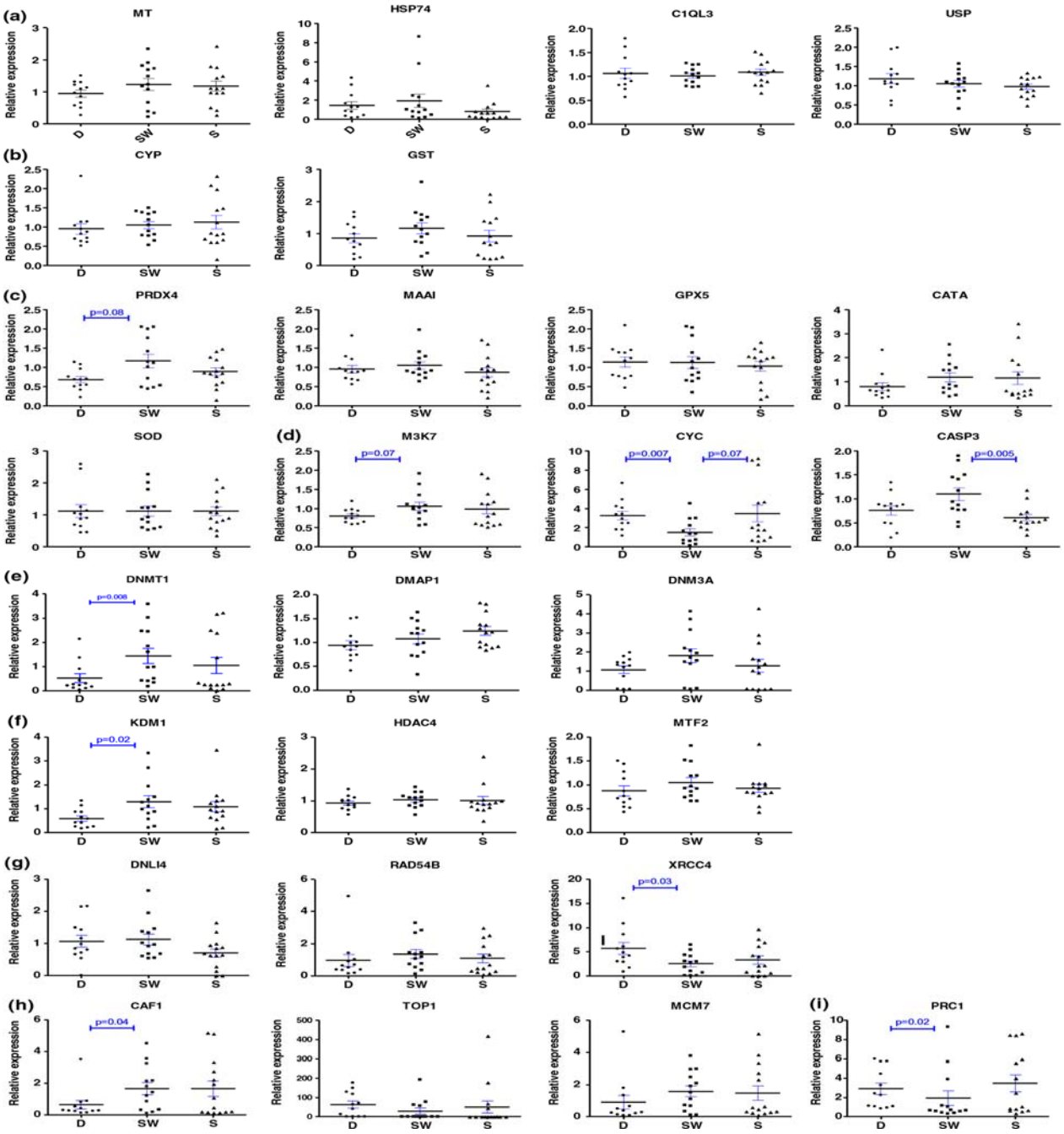

Figure 5

Expression levels of genes relevant to different functional categories in oyster genitors exposed respectively to Diuron (*D*) in comparison with seawater (*SW*) and solvent (*S*) control groups. Selected genes belong to **a** stress response, **b** xenobiotic biodegradation, **c** antioxidant, **d** apoptosis, **e** DNA methylation, **f** gene transcription regulation, **g** DNA recombinaison and DNA repair, **h** DNA replication and repair, and **i** cytokinesis. Relative expression data were normalized with the C_T geometric mean value of three reference genes (Cg-ef1α, Cg-rpl40, and Cg-rps6) according to the $2^{-\Delta\Delta CT}$ method. Significant differences in relative gene expressions between oyster groups (D, SW, and S) were determined by Mann-Whitney *U* tests and *p* values are given if significant at the $p<0.05$ threshold

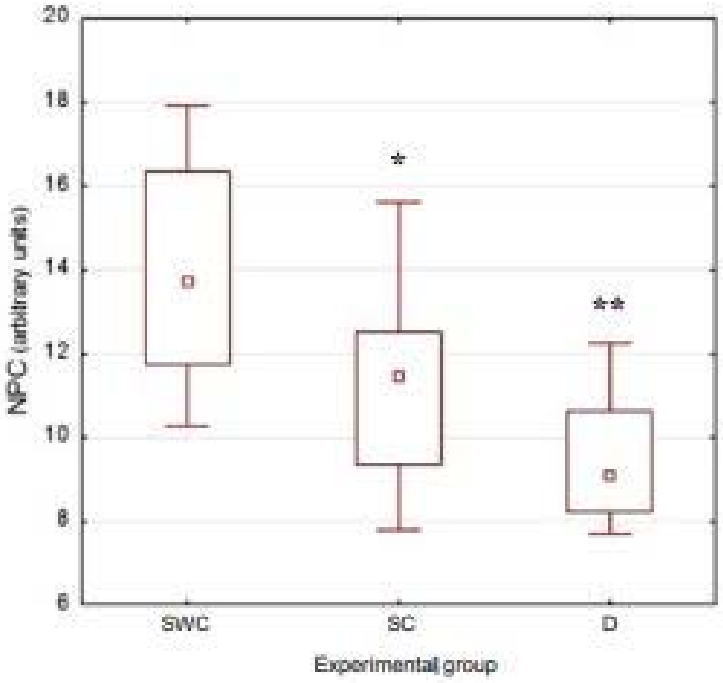

Figure 6
Normalized phagocytic capacity of hemocytes from genitor oysters measured in vitro at the end of the first exposure pulse. Plots display median value, first and third quartiles, and minimum and maximum of the data set. Significant at $*p < 0.05$ and $**p < 0.001$ (Kruskal-Wallis)